NEMATODE TECHNIQUE*

THOMAS BYRD MAGATH
Fellow in Zoology, University of Illinois

Difficulties in making satisfactory preparations for examination have prevented many from taking up problems with nematodes. The author, in his study on some of the parasitic species, undertook to solve a few of the general problems in the technique and also some of the more particular processes for demonstrating certain organs and systems. This work has by no means exhausted the problem, but perhaps the methods given here will serve as a guide for future work along this line. These methods, while applicable to free-living nematodes, have been worked out in particular for the parasitic forms.

Collecting parasitic nematodes differs little from collecting other parasitic worms. However, there are some things that have to be kept carefully in mind. In the first place many forms are encountered with such well developed mouth parts that it is difficult to free them from their attachments without injury. The best method is to take hold of the host tissue very close to the mouth of the parasite with a pair of fine-pointed forceps and with gentle pressure and slight traction, to pinch the animal off; in this procedure the forceps take up so little tissue that when the worm is freed they close with nothing between their points. When the worms are not so firmly attached, by gently stroking them with a fine camel's-hair brush in a direction away from the tissue, they will often come loose; such a brush will be found in general to be the most convenient instrument for handling parasitic nematodes.

In looking for alimentary parasites it is a safe plan to split the gut with a needle, for then the danger of cutting a worm is nil; however, in the examination of the larger animals this is not always practical and here one must very cautiously cut the wall with blunt scissors. In most cases gripping the opened gut

*Contributions from the Zoological Laboratory of the University of Illinois under the Direction of Henry B. Ward, No. 77.

tightly between the two prongs of the forceps and drawing it thru them will free, in good condition, all of the parasites that adhere. If an examination is to be made of the lungs, liver, kidneys or muscles of an animal it will be found of advantage to tease them out with needles. In case nematodes are found encysted, they should be carefully dissected out, before preservation, under a good lens or binocular.

Most nematodes are rather sensative to changes in the osmotic condition of the medium in which they are placed. Tap water has been found to be far better for temporary keeping than physiological salt solution, and while some of them live for a while in distilled water, this medium should be generally avoided. For most of the nematodes found in fresh-water fishes a 0.3% salt solution will be found to be about right. As a matter of fact the best medium to keep them in while making the necessary observations on the living material is that which collects in the dish in which the examination is being made, and into which the isolated organ has been placed for examination. In spite of the fact that Looss found the Sclerostomidae of horses and donkeys remain in good condition after being kept for hours in physiological salt solution, it will be found that there is an advantage in killing and fixing the worms as soon as possible after they have been removed from the host. Under no condition should they be allowed to dry at any stage in the process.

Owing to the nature of the cuticula nematodes are very hard to preserve. Most cold killing fluids penetrate so slowly that nematodes live for hours in fluids which will kill other parasitic worms in a few seconds; the hot fluids coagulate the proteins of their bodies before they get in, thus making penetration harder, but they have the advantage over the cold reagents in that the specimens are killed in a straight position. While this last statement is in general true, it does not always hold, for with most of the Trichinellidæ the anterior region of the body will coil up like a spring even tho the fluid be fairly hot. This may be prevented in a great measure, if not entirely, by placing the anterior end of the worm in the angle formed by pressing the points of a pair of forceps together and working the specimens to and fro in the hot fluid as

soon as placed into it. In applying this method the fluid should be first heated and then the worms transferred into it. In most species the posterior region of the males curl up, and no method has yet been devised to prevent this. No successful method of anesthetizing the worms before fixation has been found by the author, and this procedure should be avoided. If worms are cut into pieces before fixation the organs crowd out at the cut end and the whole animal presents a very abnormal picture when sectioned, due to the pulling and stretching of the tissues. However, for certain purposes this is not an objection.

Without doubt the most successful killing fluid ever recommended for general work in this group is the one devised and used by Looss. Because of the simplicity of its use and general good results, it has been followed by nearly all workers since he (1901) published the method. The procedure is this: worms, carefully freed from debris, are placed in a mixture of 20% glycerol in 70% alcohol, if the worms are small, and in 10% glycerol and 70% alcohol, if they are large, the mixture previously being heated to 80° C. They may be preserved in this mixture for future use or put into an incubator or on a paraffin bath with the cover off of the dish in which they are contained. The alcohol and water are allowed to evaporate slowly from the glycerol, care being used to keep the worms from coming into pure glycerol too quickly, else they will collapse.

Twenty-four hours is slow enough for some small worms, but twenty-four days is too fast for some of the large ascarids. The time may be regulated by the temperature or by covering the dish partially. In glycerol they are very transparent and may be studied or kept in this fluid or transferred directly into glycerine-jelly and mounted on a slide. Of course they are not stained by this process, but many characters come out beautifully in material prepared in this way. Looss transfers material directly into 96% alcohol for sectioning, after making a few incisions in the cuticula with a very fine sharp knife. They are then brought into absolute alcohol, oil of cedar and finally paraffin. He found that oil of cedar was the best medium for this purpose; on the other hand the author has had no success with it, whatsoever, but since the product as

sold commercially varies so greatly, no importance can be attached to his negative results. In connection with the very excellent work of Looss it is interesting, from the standpoint of what is to follow, to quote one of his statements: "Canada-balsam was almost entirely excluded because it made unstained objects too transparent, and stained nematodes were less favorable for mounting than unstained ones". On the same subject Braun and Lühe express themselves in no uncertain terms: "Man wirt von vornherein auf das Färben ganzer Tiere verzichten müssen und kann auch wegen der kaum zu vermeidenden Schrumpfung der Cuticula beim Aufhellen mit Kreosot, Terpentin, etc. den Einschluss in Canada-balsam oder anderen Harzen nicht anwenden".

As for general nematode technique, there seems to be in literature few references or results published other than this work of Looss, altho there are indications that other killing fluids were used to some extent by the authors prior to 1901. Since this time a method for handling nematodes was proposed by Langeron which can be used to advantage, since it is very rapid and reliable. Nematodes are killed in diluted formol (5:100) and then transferred, after several hours, into a lacto-phenol mixture, made as follows and used first in half strength:

Glycerol	2 parts
Phenol	1 part
Lactic acid	1 part
Distilled water	1 part

After a few hours the nematodes are placed in this fluid of full strength and either preserved in it, or mounted on a slide in a drop of the mixture, enclosed in a gold-size ring, covered with a cover glass, and sealed.

Looss and Braun and Lühe claim that it is not practical to mount nematodes in balsam after staining, yet when material is handled in the correct manner, it becomes the very best medium and further allows of staining to suit the needs of the student. It is obvious that animals so sensative to changes in osmotic pressure will require very cautious treatment while bringing them into paraffin or balsam, and it was Cobb who first proposed a plan by which they could be brought into these reagents, and for this purpose he

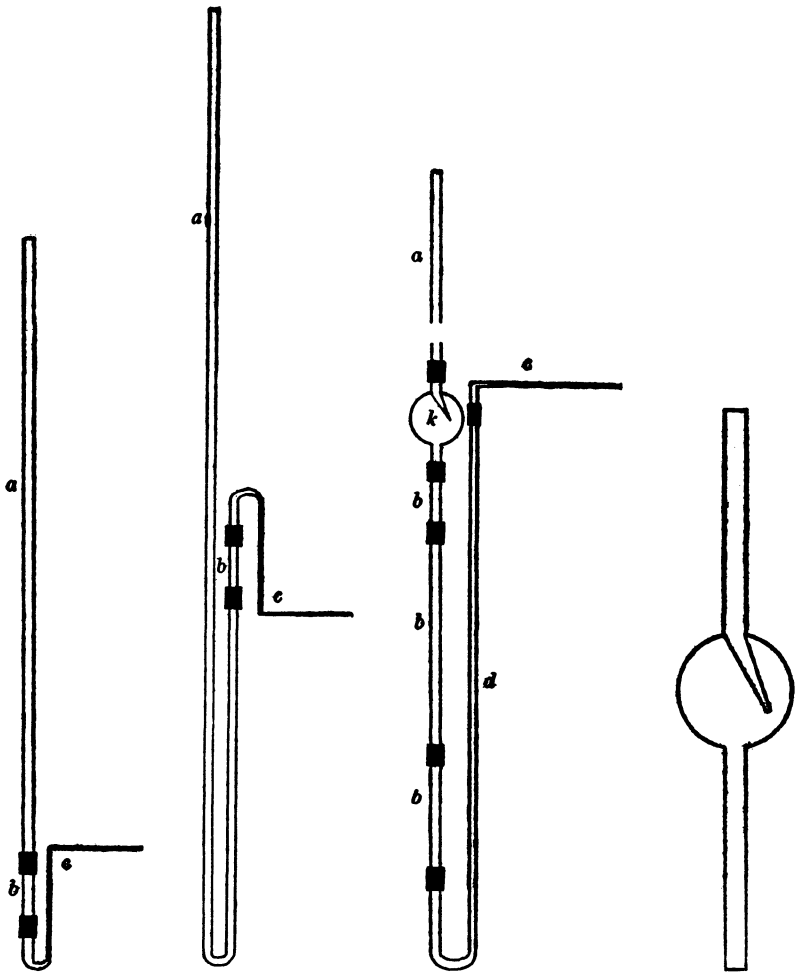

FIG. 1. FIG. 2. FIG. 3. FIG. 4.

FIG. 1. Differentiator used for dehydration. *a*, reservoir; *b*, object box, plugged at each end with cotton; *c*, filter, also with cotton plug. (After Cobb.)

FIG. 2. Differentiator used for transferring objects from absolute alcohol into a clearing fluid. Letters as in Fig. 1. (After Cobb.)

FIG. 3. New type of differentiator for dehydrating. *a*, reservoir; *b*, object holders; *c*, filter and regulation devise; *d*, safety tube; *k*, mixing chamber. The reservoir should be two metres long, and is shown sectioned in the figure. In filling, avoid bubbles.

FIG. 4. The mixing chamber of the new differentiator. Made of glass tubing, in which a bulb is blown and a pointed piece of tubing is fused into one end.

devised the differentiator. This ingenious devise (Fig. 1) consists of a glass tube 5 mm. or more in diameter, which is used for the reservoir. To this is attached, by rubber tubing, the object box, a short piece of tubing, plugged at each end with a cotton plug and holding the specimens. The object box is also attached to the filter, a third piece of glass tubing, which is drawn out into a fine capillary tip and bent as shown in the figure. The filter is filled with whatever fluid the worms are in, for instance corrosive sublimate solution, and is attached to the object box, after inserting a cotton plug in the proximal end. The box is filled and the object placed into it, the distal plug inserted and the whole connected to the reservoir, which is filled in the following manner: mix equal parts of sublimate solution and 35% alcohol (solution 2), equal parts of solution 2 and sublimate solution (solution 1), equal parts of solution 2 and 33% alcohol (solution 3). The reservoir if filled one-fourth full of each solution in order, 1, 2, 3, and then on the top 33% alcohol is added to fill the reservoir. A wire is passed down the reservoir to cause a little mixture, and withdrawn. Minute drops flow from the filter and the rate of flow can be regulated by tilting the differentiator, so that the worms are brought into 33% alcohol in from two to five hours. As the reservoir is emptied it is filled with solutions of the next higher grade of alcohol made up in the manner described. Specimens may be stained here, destained, and finally brought into absolute alcohol. Because the clearing fluids are heavier than alcohol it is necessary to use a reverse form at the tip of the differentiator. (Fig. 2). Here the pressure is equalized by bending the reservoir as figured and by the same general plan the worms are brought into the clearing fluid (Cobb used oil of cloves if the worms were uncut and chloroform if they were cut) and finally into balsam.

For some time this method was used with a fair amount of success but certain disadvantages attended it. The most obvious one is that in using the instrument so much time is required in the making up of the many solutions and the constant refilling of the reservoir. Then, too, the change is not gradual enough for the best work, especially with some very delicate worms. Further, clearing fluids attack the rubber connections. After many

trials the following method and instrument for handling the worms were devised: worms are killed in 50% alcohol heated to 60-75° C. and transferred at once to any one of the following mixtures: (A) Carnoy's (33 parts chloroform, 33 parts glacial acetic acid, 33 parts alcohol, the mixture is then saturated with corrosive sublimate), (B) a mixture made of equal parts of alcohol, water and acetic acid, saturated with corrosive sublimate, or best of all (C) using the latter mixture with the addition of enough osmic acid to make the solution contain from 0.05% to 0.1% osmic acid. These killing fluids give very good results, but for the very fine preservation of histological detail, the last one given is by far the best. In these fluids the material is left for from one to ten hours, depending upon the size of the worms. It is possible to get good results by killing in hot water and then transferring into a saturated aquous solution of corrosive sublimate, with or without acetic acid, or in combination with osmic acid. If the nematodes are killed in osmic mixtures they should be bleached by adding a little hydrogen peroxide to the water into which they are brought after fixation in water solutions, or to 50% alcohol if they are killed in alcoholic solutions. After this treatment they are brought very gradually into 70% alcohol and the corrosive sublimate removed with iodine solution. One can usually succeed in doing this within a period of ten or twelve hours and six or eight changes, so that the nematodes are perfectly round and not distorted, but to be safe, the new differentiator (Fig. 3) to be described later should be used. After the iodine is removed they are graduated into 80% alcohol and preserved in this strength, being careful not to let it become weaker. If they are to be used for totos at once they should be stained in Ehrlich's acid hematoxylin (diluted 1:25-50) or in Delafield's hematoxylin of the same dilution, in which event it will be a saving of time to remove the corrosive sublimate when they are in water or 35% alcohol with iodine dissolved in water to which a little potassium iodide is added. For sectioning it is useless to try to stain in toto, yet material may well be stained in one of the hematoxylins in order to make them easy to see and handle, or Mayer's paracarmin may be used for this purpose when they are in 70% alcohol. In either case they are stained for twenty-four hours

and then destained to the proper intensity in 5% hydrochloric acid in water or 35% alcohol and the hematoxylin material "blued" by transferring into a 5% solution of ammonia water, in either water or 35% alcohol. They are then ready for the differentiator. The new form of this instrument (Fig. 3) consists of the following parts: the reservoir, the mixing chamber, the object holders, the safety tube, and the filter and flow regulation device. The reservoir is a shell tube with an inside diameter of 5 mm. and two meters long, so that it will hold about 45 cc. of alcohol. The mixing chamber (Fig. 4) is made in the following manner: A piece of good thick-walled glass tubing, with an inside diameter of 4 mm. is pulled out at one end and sealed. Ten centimeters from the sealed end the tube is heated in a good flame and a bulb blown that will hold between six and eight cubic centimeters, then the sealed end is cut off as near the bulb as possible. The end that has been drawn out is bent in the flame near the tip (which has been broken off) at a slight angle and inserted into the bulb and the two held so that the tip points to one side and a little below the middle of the bulb; in this position they are fused together, and both ends of the tube are cut and rounded off about 5 cm. from either end of the bulb. If for any reason this type of mixing chamber cannot be made, the substitute shown in Figure 5 can be used. It is made with a piece of large glass tubing and rubber stoppers. The object holders are pieces of shell tubing with an inside diameter of from 5 to 7.5 mm. and varying in length from 5 cm. to that needed to hold the worms without bending. These are plugged with cotton at either end to keep the worms in place. The safety tube is 2 or 3 mm. inside diameter and bent in the shape of a U with one arm 5 mm. long and the other long enough to reach to the middle of the mixing chamber when in use. The filter is a tube 3 mm. in diameter, bent in the form of a right angle and pulled out into a fine capillary at one end; a cotton plug in it acts as a filter to prevent stoppage by particles in the alcohols. Rubber tubing is used for connections. To manipulate the instrument requires some practice and the order is essential. The filter is filled and connected to the safety tube as shown in Figure 3. A pinch-cock on a piece of rubber tubing at the end of the safety tube will keep it full while the rest of the

apparatus is being filled with the grade of alcohol or water in which the specimens are. The object holders are next filled and the specimens put into them and plugged with cotton. As many holders can be filled as necessary and connected together in a long line, each being properly labeled. These are then connected to the safety tube and a pinch-cock applied to the rubber-tube-capped free end. The mixing chamber is capped with a short rubber tube, then filled by drawing in the liquid of the same strength in which the specimens are, whereupon it is closed with a pinch-cock. This is then connected to the terminal object holder, the other end of the mixing chamber being connected to the reservoir. The reservoir is then filled by putting in four grades, one on top of the other, in order,

FIG. 5.

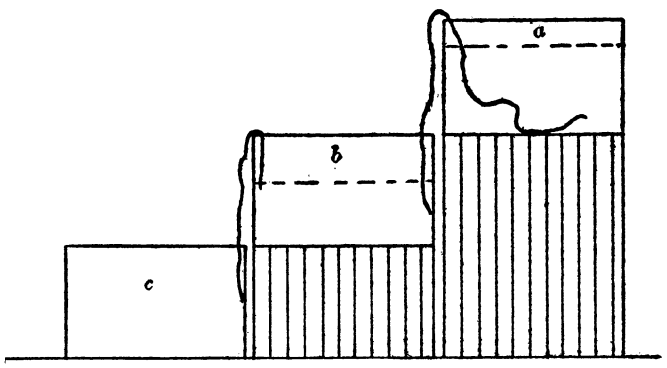

FIG. 6.

FIG. 5. Mixing chamber made of a piece of glass tubing with rubber stoppers and inlet and outlet tubes.

FIG. 6. String siphon system for handling small embryos and eggs and transferring nematodes from absolute alcohol into a clearing fluid. *a*, for the clearing fluid; *b*, for the objects; *c*, waste. *a* and *b* are supported on wooden blocks. The whole is placed under a bell-jar with sulphuric acid in a beaker to absorb the moisture.

such as 35%, 50%, 70%, 85% alcohol, the lowest being always the next step above the fluid in which the worms are contained. The capillary tip is regulated to yield 5 to 10 small drops per minute, and as the reservoir is emptied, it is filled with 95% alcohol until 20 cc. have been added. It is then filled with absolute alcohol until 50 cc. have been added, whereupon the apparatus is allowed to empty itself

as far as the safety tube will permit, as this will never allow it to run dry. The mixing chamber will attend to the mixing of the alcohols as the lighter one is forced under the heavier one and mixes very efficiently. After the absolute alcohol is all in the reservoir a calcium chloride tube is attached to the top of the reservoir, and the specimens allowed to remain in the alcohol for several hours. The object holders are now taken out ready for the clearing fluid.

The best method for getting the material into the clearing fluid is by means of a string siphon. Three Stendor dishes are arranged in stair-step fashion (Fig. 6). The objects are placed in the middle dish, either in the tubes or free, and this dish is connected with the receiving end of a siphon, made by using any suitable piece of string, regulating the flow by the size of the string. From this dish another string is led to the waste dish below. If the nematodes are to be used for sectioning one puts in the upper dish a mixture of absolute alcohol and xylol, half and half, and after this has run out xylol full strength, regulating the flow so that the specimens come into pure xylol in about 36 hours. If the specimens are intended for toto mounts synthetic oil of wintergreen (methyl salicylate) is used in the same way as xylol. The xylol-cleared worms are gradually brought into paraffin either after being cut into pieces or still whole, by allowing small pieces to dissolve in the xylol; they are finally saturated in xylol-paraffin at 35° C., and embedded after infiltration for one-half to one and a half hours in paraffin melting at 56° to 58° C. By coating, with soft paraffin, the block of hard paraffin in which the nematodes are embedded, it will be found that good sections in series can be made, provided the knife is kept sharp. For this convenient method, the author is indebted to Mr. H. G. May. Some little success has been met with infiltration in vacuo and this method, if adapted somewhat, will undoubtedly yield results worth while. The worms cleared in oil of wintergreen should be put in parchment boxes or boxes made of heavy linen bond paper and placed in Stendor dishes with damar dissolved in oil of wintergreen, and the mounting medium allowed to slowly dialyse and penetrate. It is best to pierce the cuticula in one or two places before the worms are placed into the cups, and this can be done with a sharp needle so that none of the internal organs are injured.

After twenty-four hours treatment in this manner they may be mounted under a cover glass on a slide in damar in oil of wintergreen. For staining sections good results are obtained with Delafield's hematoxylin, Ehrlich's acid hematoxylin, Unna's orcein method, Mallory's connective tissue stain and thionin in saturated solution in 1% phenol. Borax carmine is of no value and strange as it may seem, iron hematoxylin yields also poor results.

It will often be found of advantage to dissect out parts of nematodes and for this purpose glass rods drawn out into fine points are useful. For dissecting out the mouth parts, maceration in concentrated potassium hydroxide, and mounting in glycerine-jelly will give good results.

Nematode embryos and eggs are killed in strong Flemming's or the fluid of Ripart and Petit (camphor water, not saturated, 75 gms., distilled water, 75 gms., glacial acetic acid, 1 gm., copper acetate, 0.3 gm., copper chloride, 0.3 gm.) to which a few drops of osmic acid have been added, and are either carried thru the staining and dehydrating process in a differentiator or in a string siphon system. In using the latter it is best to cover it with a bell-jar and enclose also a beaker of sulphuric acid to absorbe the moisture.

Goldschmidt found that the nerve technique of Bethe, Apáthy, Wolff-Bielschowsky and Cajal could be applied to nematodes as well as to other forms. He offered a modification of the Cajal method that gave good results. Material is fixed in ammonical alcohol for 24 hours, 6 days in 10% silver nitrate in an incubator, 24 hours in hydrochinon or pyrogallol and formol; it is then embedded and sectioned. The sections are treated for twenty minutes in 0.1% goldchloride and reduced in a sodium fixing bath for half an hour. His method of preparing toto mounts of the large forms to show the nervous system was to split the cuticula and remove the esophagus. The stretched out "shell" was then stained for 6 to 8 hours in Nissl's alkaline methylene blue at 60° C., dehydrated and cleared in oil of cloves, in which they were kept, the excess stain being taken out by the action of the oil of cloves in two or three days.

The methods herein described have given good results for the author and it is hoped that others will be able to reproduce and

improve upon them. Several failures should not discourage any one, for at best time is required to obtain really good results in this very difficult field.

The author wishes to express his sincere thanks to Professor Henry B. Ward for his careful suggestions and criticism of the manuscript of this article. Valuable assistance in many ways has been received thru the close personal association with my friend Mr. Henry G. May.

REFERENCES

BRAUN, M. UND LÜHE, M.
1909. Leitfaden zur Untersuchung der tierischen Parasiten des Menschen und der Haustiere. Würzburg. [pp. 95-97, 98, 100.]

COBB, N. A.
1890. Two new Instruments for Biologists. Proc. Linn. Soc. N. S. Wales, (2) 5:157-167, 1 pl. Reviewed in Jour. Royal Micr. Soc., 1890:821-822, 2 figures.

GOLDSCHMIDT, R.
1908. Das Nervensystem von Ascaris lumbricoides und megalocephala. I Teil. Zeit. wiss. zool., 90:73-136, 4 pl.
1910. Das Nervensystem von Ascaris lumbricoides und megalocephala. III Teil. Festschr. sechzigsten Geburtstage Rich. Hertwigs, 2:255-354, 23 pl.

HASWELL, W. A.
1891. On a Simple Method of Substituting Strong Alcohol for a Watery Solution in the Preparation of Specimens. Proc. Linn. Soc. N. S. Wales, (2) 6:433-436, 1 figure. Reviewed in Jour. Royal Micr. Soc., 1892:696-698, 1 figure.

LANGERON, M.
1905. Note sur l'emploi du lactophénol de Amann pour le montage des nématodes. C. R. Soc. Biol., 58:749-750.

LEE, A. B.
1913. The Microtomist's Vade-Mecum. 7th Ed. [pp. 224 and 398.]

LOOSS, A.
1901. The Sclerostomidæ of Horses and Donkeys in Egypt. Records of Govt. School of Medicine 1901:27-138, 13 pl.

RANSOM, B. H.
1911. The Nematodes Parasitic in the Alimentary Tract of Cattle, Sheep, and other Ruminants. Bur. Anim. Ind. Bull. 127.

STITT, E. R.
1912. A quick Method for accurately Differentiating the species of Hookworm of Man. Jour. Amer. Med. Assoc., 59:1706-1707.